T. BOUDIERI EXTRA CT POTENTIATES THE EFFECTS OF CAPECITABINE TREATMENT IN HUMAN COLON CANCER CELLS

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1. INTRODUCTION

Colorectal cancer is one of the most frequently occurring types of cancer worldwide, ranking as high as second in certain regions in terms of incidence, with a projected increase in mortality rate of 66% by the year 2035 (Sawicki et al., 2021). This predicted rise in colorectal cancer cases can be attributed to a wider spread in multiple factors implicated in the development of this type of cancer, such as increased sedentary life-styles and unhealthy diets, what is more concerning however is the increase in incidence of early-onset colorectal cancer in individuals below the age of 50 (Akimoto et al., 2021). In addition to its high incidence, colorectal cancer also has a high mortality rate, constituting in some countries the third or even second cause of cancer related deaths (Siegel et al., 2020). Another reason that highlights the urgency for continuing to explore anti-cancer chemotherapeutic alternatives that can act as stand-alone treatments or adjunct therapies as well as synergistic tools to boost the efficacy of chemotherapeutic agents already being utilized is that cancer cells generally employ several mechanisms to develop drug resistance to known treatments and evade them such as mutations in various proteins involved in cell signaling pathways that are targeted by chemotherapeutic agents (Nussinov, Tsai, & Jang, 2021).

The natural world is filled with bioactive substances that have demonstrated a capacity to influence and regulate key proteins in the human body involved in cell signaling pathways that drive various aspects of human physiology such as immunity and cancer. Plants as well as fungi are rich in nutrients important not only for sustenance, but also for their medicinal properties (Silva & Fernandes Júnior, 2010). Bioactive compounds from natural sources have demonstrated a capacity to act as anti-inflammatory, anti-microbial, and antiproliferative agents when it comes to cancer cells, as well as act as excellent supplements to ameliorate the negative side effects of drugs with toxic side effects (Goyal, Gupta, Chatterjee, & Nimesh, 2017; Mueller, Hobiger, & Junghauer, 2010; Rahman et al., 2021; Reda, Borjac, & Usta, 2020; Zahrddin, Khalil, & Hijazi, 2022). Indeed, we have been part of the effort to demonstrate that *Terfezia boudieri*, a desert truffle that is widely available in our geographical region, and a dietary staple for many, has the capacity to regulate several key proteins that lie at the heart of cellular apoptosis, cell proliferation, and the development and metastasis of cancer cells. *T. boudieri* water extract was able to decrease the proliferation of HCT-116 and Caco-2 colon cancer cells, reduce their migration capacity, and induce apoptosis. Western Blot studies revealed a reduction in the levels of Bcl-2, c-Myc, and cyclin D1 and an increase in the levels of p53 and Bax (Sawaya, Abou Najem, Khawaja, & Khalil, 2023). Bcl-2 is a major player in the apoptotic pathway of programmed cell death which acts by suppressing it through inhibiting the pro-apoptotic function of Bax, furthermore conferring resistance to chemotherapy (Antonsson & Martinou, 2000; Hockenbery, 1994). An important human oncogene frequently found in many types of cancer, c-Myc acts not only as a proliferation driver which increases cellular metabolism and differentiation, but also enhances cell-mediated angiogenesis by colon cancer cells (Chen et al., 2013). Cyclin D1 is involved in the progression of the cell cycle, driving the progression from the G1 to the S phase, and acts as an important prognostic marker in colorectal cancer patients (Bahnassy et al., 2004). On the other hand, Bax, which belongs to Bcl-2 family of proteins is an important driver of apoptosis, the mechanism by which cells undergo programmed death, thereby preventing the development of cancer, and higher levels of Bax correlate with better prognosis for colorectal cancer patients (Katkoori et al., 2010). Last but not least, p53, dubbed the guardian of the genome is an important tumor suppressor that has become a target of interest in the treatment of cancer as it plays a role cell cycle arrest, apoptosis, control of genome integrity, and DNA repair, to name a few, its
integrity so important that the TP53 gene has been found to be mutated in most cancers and more specifically in approximately 60% of colorectal patients (Agarwal, Taylor, Chernov, Chernova, & Stark, 1998; Levine, 2020; Michel, Kaps, Maderer, Galle, & Moehler, 2021).

Several analyses geared towards uncovering the compounds found in T. boudieri have demonstrated that it has a high nutritional value and that it contains an array of important minerals as well as bioactive compounds with strong anti-oxidant properties such as phytosterols, flavonoids, tannins, and terpenoids (Akyüz, 2013; Dundar et al., 2012).

In addition to the ability of plant-derived phytosterols to regulate blood lipid profile, studies on a number of cancer cell lines such as ovarian, breast, and liver, lung, prostate, and stomach, have demonstrated that they have anti-cancerous properties which they allegedly exert through inhibition of cancer cell multiplication as well as invasion and metastasis. That, in addition to the induction of cell cycle arrest and stimulation of apoptosis (Blanco-Vaca, Cedó, & Julve, 2019; Zhong et al., 2018). Flavonoids, which comprise the biggest group of plant polyphenols can act as anti-inflammatory agents by regulating the levels of NF-κB in various ways, thus ameliorating the inflammatory process in cancer cells. They can also act as antioxidant suppressors of reactive oxygen species, modulate cell growth through influencing a series of cell signaling pathways relevant to cellular growth. For example, they can inhibit the AKT/mTOR and Ras/ERK signaling pathway, thereby impairing cancer cell metabolism, proliferation, and even angiogenesis (Asati, Mahapatra, & Bharti, 2016). Interestingly, studies have also suggested that flavonoids have an important preventative role when it comes to cancer development through the same molecular mechanisms (Romagnolo & Selmin, 2012).

Tannins are another plant-derived group of biomolecules with strong evidence from multiple studies that indicate an important anti-cancerous capacity, but also the ability to help overcome multi-drug resistance in cancer cell treatment, making them a potentially valuable agent that can be used as a chemotherapeutic adjuvant (Kleszcz, Majchrzak-Celińska, & Baer-Dubowska, 2023). Last but not least, terpenoids have also been receiving focus for having demonstrated anti-cancerous properties through the induction of cancer cell autophagy via complex signaling pathways such as MAPK/ERK/JNK, PI3K/AKT/mTOR, AMPK, NF-kB, and reactive oxygen species (Chopra, Dhingra, Dhar, & Nepali, 2021; El-Baba et al., 2021)

Considering the aforementioned with regards to the anti-cancerous properties of T. boudieri water extract it was of interest to investigate whether this extract can act as a potentiator for other therapeutic drugs when used concomitantly.

Capecitabine is an anti-metabolic drug that acts by disrupting DNA and RNA synthesis leading to cell death after being converted to its active 5-FU form through three sequential enzymatic reactions (Hirsch & Zafar, 2011). One of the most mainstream chemotherapeutic agents used against colorectal cancer, capecitabine is utilized either as a monotherapy or in combination with other drugs (Pouya, Rasmi, Camci, Tutar, & Nemati, 2021).

We attempt in this study to explore whether the water extract of T. boudieri has the potential to act synergistically with capecitabine on the human colon cancer cell line HCT-116, thereby increasing its efficacy.
2. MATERIALS AND METHODS

2.1. Extract Preparation.
Black desert truffle obtained from the Lebanese local market were properly cleaned by scrubbing, which was also used to dispose of their peel. 250 grams of truffles were sliced thinly and air dried at 37°C. Dry crusts were then ground into powder that ended up weighing 35 grams, and soaked in distilled water for 24h (1:4 ratio w/v). Whatman filter paper was used for filtration to ensure that the water extract had no residual pieces. The extract was then lyophilized into a soft aromatic paste that can be dissolved in the cell culture medium to prepare different concentrations for treatment. DMEM - Dulbecco's Modified Eagle Medium (Lonza, OR, USA) was used as a solvent and filtered using a 33 mm syringe filter (Sigma-Aldrich, Germany).

Yield (%) = [Amount of lyophilized water extract (g) / amount of dried matter (g)] x 100

2.2. Cell Maintenance.
HCT-116 cells purchased from the American Type Culture Collection (Manassas, VA, USA) were gifted from the Cell Culture Facility of the Faculty of Science, Alexandria University, Egypt. Cells were cultured with complete Dulbecco’s modified eagle medium (DMEM) (Lonza). The DMEM contained 10% FBS (Sigma-Aldrich), 1% L-glutamine (Sigma-Aldrich), and 1% antibiotic-antimycotic (Biowest, Bradenton, FL, USA). Cells were incubated at 37°C in 5% CO2 and 95% humidity. The 9th and 10th passages were used.

2.3. Cell Viability Assay.
Ninety-six well plates were incubated for 24 h with 15 x 10^3 cells per well and left until 60-70% confluency. Different concentrations of the water extract of T. boudieri (50-3.12 mg/ml) were then applied, other wells were treated with different concentrations of capecitabine (100-6.25 µM), or a combination of different concentrations of both. These extract concentrations were selected based on previous MTT assay trials at our lab to determine the most optimal range range, while those of capecitabine were extrapolated from available literature (M. Li, Zhang, & Li, 2017). All treatments were applied in triplicates and left for another 24 hrs. Afterwards the culture media were replaced by a new complete media in each well in addition to 10 µl of MTT solution. This solution was prepared by dissolving 5 mg of MTT powder (Sigma-Aldrich) in 1 ml of 1x PBS (Sigma-Aldrich) and filtered with a 33mm syringe filter (Sigma-Aldrich). The plates were then incubated in complete darkness at 37°C for 4 hrs. 100 µl of isopropanol were then added to each well and the plate was left overnight in order to dissolve the formazan crystals. A microplate reader was used to measure the optical density at 595 nm (Multiskan FC, Sigma-Aldrich, Germany).

2.4. Combination Index Analysis.
To determine whether combining the water extract with capecitabine potentiated the anti-cancerous effects of the latter, data from the cell viability assay were analyzed using the CompuSyn software (ComboSyn, Inc.). This analysis relies on the Chou and Talalay's equations to calculate the combination index (CI) of drugs using the following equation (Chou & Talalay, 1983):

\[ CI = \frac{(D_1)}{(D_{x1})} + \frac{(D_2)}{(D_{x2})} \]

Here \( (D_1) \) and \( (D_2) \) are drug doses of the first and second drug when combined together to inhibit a system x percent and where \( (D_{x1}) \) and \( (D_{x2}) \) are the drug doses of the drugs that were given individually and inhibited the system x percent. According to the software, \( CI \leq 1 \) means there is synergism, \( CI = 1 \) implies an additive effect, and \( CI \geq 1 \) indicates an antagonism between the drugs.
2.5. Cytomorphological Changes.
In order to observe the morphological changes in treated cells as well as their density in the wells, 96 well plates with treated cells were dyed with 0.5% crystal violet for 30 mins and washed with tap water. Images of the wells were taken under the microscope at 200x magnification.

2.6. Statistical Analysis
Data from the cell viability assay were analyzed using mean ±SEM (Standard Error of the Mean). GraphPad Prism version 9 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) was utilized. IC$_{50}$ was obtained using non-linear regression. A p value <0.0001 denoted statistically significant differences.

3. RESULTS
3.1. Percentage Yield of the Water Extract
The water extract resulted after lyophilization in a very aromatic and soft brownish paste. The total yield was around 12%.

3.2. Effect of the Water Extract on Cell Viability in HCT-116 Cells.
HCT-116 cells treated with the water extract demonstrated a dose dependent anti-proliferative effect in accordance with our previous studies. As the concentration of the water extract applied rose, the percent cell viability diminished, with an IC$_{50}$ of 6.6 mg/ml. This percent cell viability was further reduced in cells treated with a combination of capecitabine and the extract when compared to treatment with the drug alone, leading to a reduction in IC$_{50}$ (as indicated in Table 1). This drop in the percentage of cell viability was statistically significant in HCT-116 when the doses used were 12.5 mg/ml of extract with 20 µM of capecitabine, compared to 20 µM of capecitabine alone (as shown in Fig 1). 5% crystal violet applied for 30 mins on a 96 well plate containing treated cells allowed for visualization of the effect of different treatments, their concentrations alone, and combined concentrations (as shown in Error! Reference source not found.). The results of the crystal violet assay were consistent with those of the cell viability assay indicating that when combined with the water extract the apoptotic potential of capecitabine was higher than when it was used as a stand-alone treatment. This potentiation of the anti-cancerous effect of capecitabine treatment was reflected in the MTT assay as a drop in the IC$_{50}$ value when cells were treated with the drug alone as compared to the combination of drug-water extract, while in the crystal violet assay this potentiation was translated as a clearly visible reduction in cellular density. Wells that were treated with a combination of capecitabine and water extract stained lighter as compared to the more densely stained wells where the cells were treated with the drug alone, as live cells that remain adherent to the bottom of the wells display a violet stain (as shown in Fig 2).

3.3. Effect of the Combined Treatment as a Measure of the Combination Index (CI).
CompuSyn was used to analyze the absorbance values obtained from the MTT assay, results showed that the extract does have a synergistic effect when combined with capecitabine, specifically at a concentration of 25mg/ml of extract and 50 µM of capecitabine resulting in a CI index of 0.83 whilst a CI < 1 is indicative of synergy.
Fig 1. Percent viability of HCT-116 cells treated with different concentrations of the water extract alone, in combination with capecitabine, or different concentrations of capecitabine alone. Showing a reduction in combination IC$_{50}$ compared to drug alone and a dose dependent cytotoxic effect for the water extract.

Table 1. The half-maximal inhibitory concentration (IC$_{50}$) for HCT-116 treated with the extract, capecitabine, and their combination.

<table>
<thead>
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<th>IC$_{50}$ ± SEM</th>
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<tr>
<td>Capecitabine (µM)</td>
<td>67.54 ± 4.98</td>
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<tr>
<td>Extract (mg/ml)</td>
<td>6.6 ± 0.12</td>
</tr>
<tr>
<td>Extract + Capecitabine (mg/ml + µM)</td>
<td>8.2 ± 0.36 16.59 ± 0.73</td>
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Fig 2. Crystal Violet assay showing decreased staining density in wells with lower cell viability as a reflection of the cytotoxic effect on HCT-116 cells exerted by a range of concentrations of the extract alone, capecitabine alone, and a combination of both. It shows a reduction in cell viability in wells where cells were
treated with a combination of capecitabine and extract when compared to those treated with capecitabine alone at all concentrations.

Microscopic images at 200x magnification were obtained of the wells where cells had undergone different treatments either with the extract or capecitabine alone or in combination at different concentrations. Key morphological features of apoptosis such as cellular shrinkage and darkening were more distinctive in the wells where HCT-116 cells were treated with a combination of both the extract and capecitabine. All wells showed reduced number of cells compared the to control wells.

![Microscopic images showing cellular shrinkage and darkening in treated cells](image)

Fig 3. Control HCT-116 cells as well as cells treated with IC$_{50}$ of capecitabine (Cap). IC$_{50}$ of the combination of capecitabine and the extract (Cap + Ext), and IC$_{50}$ of the extract alone (Ext) are displayed above. Shrinkage and nuclear darkening as a result of chromatin condensation, both hallmark signs of apoptosis, were more prominent in cell lines treated with the combination of the extract and capecitabine as compared to those treated with capecitabine alone. Reduced number of cells was also clear, especially in comparison to the control where the cells are arranged in a uniform layer, but more clearly where HCT-116 cells were treated with the combination.

4. DISCUSSION AND CONCLUSION
In light of the ability of cancer cells to possess or develop de novo resistance to multiple chemotherapeutic drugs there is an increasing interest in the adapting a poly-drug approach in the treatment of cancer patients. Over 90% of death in cancer patients receiving treatment with conventional chemotherapeutics has been attributed to multidrug resistance by cancer cells (Bukowski, Kciuk, & Kontek, 2020).

In addition to drug resistance, conventional chemotherapeutic drugs are for the most part toxic agents which have various negative side effects some which can persist for life (van den Boogaard, Komninou, & Vermeij, 2022). This has pushed the research for uncovering alternative treatment options towards natural products extracted from plants and mushrooms. Natural extracts contain compounds and secondary metabolites which have now been well documented to have anti-cancer properties (Abdalla et al., 2022). In their paper Rayan et al resent the case that “nature is the best source of anti-cancer drugs” due to the large number of natural compounds that have been screened for anti-cancerous properties and found to possess it (Rayan, Raiyn, & Falah, 2017).
Not only do bioactive compounds from natural sources of plant and mushroom material possess anti-cancerous and anti-inflammatory properties, but they also present strong antioxidant effects and convey physiological benefits thereby supporting the well-being and nutritional demands of consumers. These benefits can help ameliorate the negative side effects and toxicity of mainstream chemotherapeutics when used in combination with natural compounds (Lin et al., 2020). In fact, a study by Nouiri et al conducted in rats found that the water extract of *T. boudieri* has a protective as well as curative effect against paracetamol acute toxicity on the kidneys and livers of the rats (Nouiri et al., 2021). Considering its documented antioxidant, hepatoprotective, anti-cancerous and anti-angiogenic effects, as well as its high nutritional profile, the water extract of *T. boudieri* makes an excellent candidate to be investigated as an addition to conventional chemotherapeutic treatments that might be able to potentiate the efficacy of these drugs, and possibly protect against their negative side effects (Dundar et al., 2012; Nouiri et al., 2021; Sawaya et al., 2023).

Our cell viability assay using MTT on HCT-116 cells treated with either the water extract of *T. boudieri* alone, in combination with capecitabine, a commonly used first line of treatment drug in colorectal cancer, or capecitabine alone has demonstrated results aligned with our previous study, that the water extract does have a cytotoxic effect on colon cancer cells, but also in addition to that, that percent viability diminished in cells treated with the combination of extract and capecitabine compared to those where capecitabine was used alone, bringing the IC\(_{50}\) of the drug down from 67 µM to 16.69 µM when in combination with 8.5 mg/ml of the water extract. This potentiation, however, goes further than an additive effect. Using the combination index, we have demonstrated that the extract possesses a synergistic effect when added to capecitabine reflected in a CI of 0.83 when 25 mg/ml of extract were added to 50 µM of capecitabine.

The potentiation effect could be observed by the naked eye when 96 well plates with treated HCT-116 cells were stained with crystal violet. Wells where the cells had undergone combined treatment appeared much less stained as the viable cells that remained attached to the bottom of the wells decreased in number extensively in wells containing cells subjected to the combined treatment. Cytomorphological features observed under the microscope of the HCT-116 cells stained with crystal violet reflected higher rates of apoptosis in cells with the combined treatment, more so than in capecitabine alone, although all treated cells had evident apoptosis compared to control. These apoptotic features were cellular shrinkage and darkening of the nucleus.

These results could be attributed to the sum of bioactive constituents of *T. boudieri*, as they have individually demonstrated anti-cancerous effects through influencing a plethora of cell signaling pathways that deal with cancer cell proliferation, cell cycle progression, and apoptosis, as previously mentioned. Studies on molecules such as flavonoids have not only demonstrated their anti-cancerous capacity, but also highlighted their potential value as candidates for synergistic chemotherapy due to their ability to hinder molecular mechanisms exploited by cancer cells that are responsible for multidrug resistance (Hussain, Luqman, & Meena, 2020). The same applies to tannic acid which has been demonstrated to act synergistically with doxorubicin, increasing its intracellular bioavailability in colon cancer cells Caco-2 (H. Li, Krstin, & Wink, 2018). We have also previously demonstrated that the extract leads to reduction in levels of c-Myc and cyclin D1. Studies involving both c-myc and cyclin D1 inhibitors have shown that such molecules can act in synergy with other chemotherapeutic agents (Huang, Weng, Zhou, & Qu, 2014; Wu et al., 2002).

In conclusion, our study reveals that the water extract of *T. boudieri* has the capacity to potentiate the effect of capecitabine, a mainstream chemotherapeutic and a first line of treatment drug in colorectal cancer, beyond an additive anti-cancer effect, acting synergistically to induce a higher level of cytotoxicity. This makes it an excellent candidate to further investigate and consider in polydrug approaches to cancer therapy. We suspect, based on the studies and profile of the extract that the positive side effects it can introduce to chemotherapy surpass its capacity to act as a
synergistic drug, possibly capable of ameliorating negative side effects of accompanying drugs and acting to protect patients’ organs against toxic side effects while also providing a good nutritional supplement.

References


